DATA EVALUATION RECORD

DICAMBA

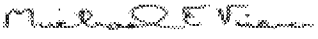
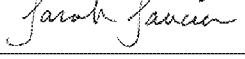
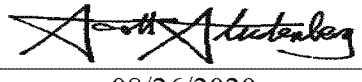
Study Type: OCSPP Non-Guideline; Mechanistic Follow-Up Study in Rats

EPA Contract No. EP-W-16-018
Task Assignment No. 34-3-001 (MRID 51129103)

Prepared for
Health Effects Division
Office of Pesticides Program
U.S. Environmental Protection Agency
2777 South Crystal Drive
Arlington, VA 22202

Prepared by

10560 Arrowhead Dr., Suite 500
Fairfax, VA 22030

Primary Reviewer:	Signature:	
Michael E. Viana, Ph.D.	Date:	08/11/2020
Secondary Reviewer:	Signature:	
Sarah E. Saucier, Ph.D.	Date:	08/24/2020
Quality Assurance:	Signature:	
Scott D. Studenberg, Ph.D., DABT	Date:	08/26/2020
Project Manager:	Signature:	
Michael E. Viana, Ph.D.	Date:	08/31/2020

This Data Evaluation Record may have been altered by the Health Effects Division subsequent to signing by CDM/CSS-Dynamac Joint Venture personnel. Contractor's role did not include establishing Agency policy.

EPA Reviewer: Sarah Dobreniecki **Signature:** Sarah Dobreniecki
Risk Assessment Branch VII, HED (7509P) **Date:** 9/16/2020
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DATA EVALUATION RECORD

STUDY TYPE: Mechanistic Follow-Up Study in Rats; OCSPP Non-Guideline; OECD 489.

PC CODE: 029801

DP BARCODE: D458715

TXR #: 0058082

TEST MATERIAL (PURITY): Dicamba (89.8% a.i.)

SYNONYMS: BAS 183 H; SAN837; 3,6-dichloro-2-methoxybenzoic acid

CITATION: Barfield, W. (2020) Dicamba techn. (BAS 183 H; SAN837 techn.): Follow up study to determine potential *ex vivo* effects during comet tissue processing. Covance CRS Ltd., Alconbury, Huntingdon, Cambridgeshire, UK. Laboratory Study No.: MM44NB, March 30, 2020. MRID 51129103. Unpublished.

SPONSORS: Syngenta, Ltd., Jealott's Hill International Research Centre, Bracknell, Berkshire, UK

BASF SE, Ludwigshafen, Germany

EXECUTIVE SUMMARY: In a concurrently-reviewed, non-guideline, *in vivo* Comet test (MRID 51129101), increased DNA strand breaks accompanied by increased numbers of hedgehog cells were observed in the duodenum of Crl:CD(SD) male rats administered dicamba in aqueous 0.5% methylcellulose via oral gavage (dose volume 10 mL/kg) at dose levels of 37.5 or 75 mg/kg/day. In order to further investigate potential *ex vivo* effects on point-of-contact tissues, the present study was performed. In this non-guideline, mechanistic follow-up study (MRID 51129103), groups of three Crl:CD(SD) male rats were administered dicamba (89.8% a.i., batch # P.MG2726410) in aqueous 0.5% methylcellulose via oral gavage (dose volume 10 mL/kg) at dose levels of 0 or 75 mg/kg/day; two doses were administered approximately 24 hours apart. At approximately 0.5, 1, 2, 4, or 6 hours after the second dose, the rats were euthanized; control rats were euthanized 2 hours after the second administration of vehicle. A seventh group of three rats were administered three doses at 25 mg/kg at 30-minute intervals; this dosing was performed twice 24 hours apart, and the rats were euthanized 2 hours after the second dosing was completed. A positive control group was administered ethyl methanesulfonate in purified water at 200 mg/kg and euthanized 3 hours after the single dose. Single cell suspensions of the duodenum and liver were prepared for comet analysis.

Comet test data showed increased % tail intensity accompanied by increased hedgehog cells across some, but not all, time points. The positive control generated data consistent with expected findings. Intracellular pH data was subjective, not quantifiable, and inconclusive.

Mincing solution pH and osmolality was not affected by incubation times or cell suspension preparation. There were no microscopic findings of cytotoxicity, necrosis, or apoptosis.

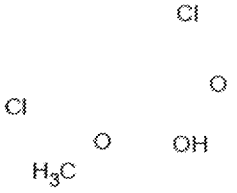
This study is classified as **acceptable / non-guideline**.

COMPLIANCE: Signed and dated Data Confidentiality, GLP Compliance, Flagging, and Quality Assurance statements were provided.

I. MATERIALS AND METHODS

A. MATERIALS

1. Test material: Dicamba

Description:	White solid
Batch #:	P.MG2726410
Purity:	89.8% a.i.
CAS # of TGAI:	1918-00-9
Stability:	Approximately two years stored at <30°C
Structure:	

2. Vehicle: Aqueous 0.5% (w/v) methylcellulose (batch #: SLBR8963V; Sigma).

3. Test animals

Species:	Rat (male only)
Strain:	Crl:CD(SD)
Age / weight at Day 1:	Approximately 7-8 weeks / 218-250 g
Source:	Charles River UK Ltd. (Margate, Kent, England)
Housing:	Three rats/cage; however, additional information (type of cage) were not provided. Rats were provided untreated wood chew blocks and a red plastic shelter for environmental enrichment.
Diet:	Pelleted Envigo Teklad 2014C diet, *ad libitum*
Water:	Tap water, *ad libitum*
Environmental conditions	
Temperature:	20-24°C
Humidity:	40-70%
Air changes:	Not provided
Photoperiod:	12 hours light/12 hours dark
Acclimation period:	5 days minimum

B. STUDY DESIGN

1. In-life dates: Not reported

2. Animal assignment: Following receipt, the rats were weighed and randomly assigned to the groups in Table 1. No additional information was reported.

TABLE 1: Study design [a]

Group	Treatment	Concentration (mg/mL)	Dose (mg/kg/day)	Time of tissue sampling (hours) [b]	# of rats
1	Vehicle	0	0	2	3
2	Dicamba	7.5	75	0.5	3
3	Dicamba	7.5	75	1	3
4	Dicamba	7.5	75	2	3
5	Dicamba	7.5	75	4	3
6	Dicamba	7.5	75	6	3
7	Dicamba	2.5 [c]	75	2	3
8	EMS [d]	20	200	3	3

a Data were obtained from page 20 of MRID 51129103.
b Time after the second or final dose on Day 2.
c Rats were administered three doses of 25 mg/kg at 30-minute intervals on two consecutive days.
d EMS = ethyl methanesulfonate, single 200 mg/kg dose.

3. **Dose-selection rationale:** The 75 mg/kg/day dose was used in the initial comet test (MRID 51129101).

4. **Preparation of test formulations:** For each dose formulation, an appropriate amount of the test substance (adjusted for purity) was weighed and ground in a mortar with a pestle; vehicle (aqueous 0.5% w/v methylcellulose) was added and mixed to form a paste. Additional vehicle was added to produce a smooth, pourable suspension, and the suspension was brought up to final volume with vehicle and homogenized.

   Homogeneity and stability analyses were reported in MRID 51129101; the results are reported here. Concentration analyses were not reported. It was stated that the pH of the 2.5 mg/mL formulation was 2.49 and the pH of the 7.5 mg/mL formulation was 2.10.

5. **Dose administration:** The dose suspensions and positive control were administered by oral gavage at a dose volume of 10 mL/kg; the volumes were calculated from the most recent body weights. The dose suspensions were administered twice approximately 24 hours apart.

6. **Statistics:** Statistical analyses were not reported. The data generated were compared to historical control data.

C. METHODS

1. **Observation:** The rats were observed regularly throughout the working day for mortality and morbidity.

2. **Body weight:** All rats were weighed following arrival, on each day of dosing, and at euthanasia.

3. **Food consumption:** Food consumption was not reported.

4. **Euthanasia:** The rats were euthanized by carbon dioxide asphyxiation at the times indicated in Table 1 (after the second or final dose on Day 2).

5. **Single cell preparations:** Two methods for preparation of single cell suspensions from the duodenum samples were used. Comet slides were prepared from the duodenum of animals in Groups 1 to 8 by using Method 1 and Groups 1 to 6 by using Method 2. Single cell suspensions of liver samples also were prepared and used for intracellular pH measurements.

a. **Duodenum method 1:** The duodenum was excised, cleaned with complete mincing solution (not described) by using the back of a scalpel blade, and placed into a tube with additional mincing solution. The samples were incubated on ice for approximately 30 minutes (± 10 minutes) and cleaned a second time as above. The tissue was transferred to a dish, additional complete mincing solution was added, and the tissue was minced by using a scalpel blade to release the cells. The cells were stored in a tube on ice until slide preparation.

b. **Duodenum method 2:** The duodenum was excised, placed in ice-cold Merchants solution (not described), and vortexed for 15 seconds. The tissue was moved to a petri dish and the inner surface scraped twice with the back of a scalpel blade to remove any waste material. The tissue was moved to fresh ice-cold Merchants solution, vortexed for 15 seconds, and placed back in the dish. Ice-cold Merchants solution (150 µL) was pipetted onto each duodenum and the inner surface scraped three times (the Reviewers assume this step released the cells). The cell suspension was stored in a tube.

c. **Liver suspensions:** Small sections of liver (approximately 0.5 cm^3) were cut and washed in fresh complete mincing solution (not described) to remove as much blood as possible. Fresh mincing solution was added, and the liver section was cut into several smaller pieces. The pieces were transferred to a portion of 150-µm bolting cloth (not described) placed over a tube and the liver was pushed through the cloth. Additional mincing solution was added and any remaining liver was pushed through the cloth. The cell suspension was stored on ice until slide preparation.

6. **Intracellular pH measurements:** Intracellular pH was measured with two commercially-available kits (Fluorometric Intracellular pH Assay Kit, Sigma [kit 1] and pHrodo™ Green AM intracellular pH Indicator, ThermoFischer [kit 2]). Briefly, the remaining cell preparations obtained from the duodenum by using Method 1 were divided into two equal portions and centrifuged. Two portions (500 µL) of the liver cell preparations were centrifuged and the supernatant discarded. For kit 1, the cell pellets were stained with the dye reagent (100 µL) and incubated for 30 minutes at 37°C in a 5% CO_2 atmosphere. The cells were qualitatively assessed for pH with a fluorescence microscope (λ excitation = 490 nm / λ emission = 535 nm). It was stated that the dye used (BCFL-AM; not described) penetrated into the cells. An acetoxymethyl (AM) group was cleaved by intracellular esterase hydrolysis and the resulting BCFL fluorophore was retained in the cell. The fluorescence intensity of the dye peaks at neutral pH and is reduced by lowering the pH. Thus, a reduction of the fluorescence is a signal for low intracellular pH values. For kit 2, the cell pellets were washed once in LCIS buffer (not described) and centrifuged again. The dye reagent (100 µL) was added to the pellet, the pellet was resuspended, and the suspension was incubated at 34-39°C for 30 minutes. The cells were then qualitatively

assessed for pH with a fluorescence microscope (λ excitation = 490 nm / λ emission = 535 nm). The dye used for this stain is weakly fluorescent at neutral pH but increasingly fluorescent as the pH drops.

7. **Mincing solution pH and osmolality measurements:** The pH and osmolality of the mincing solution were determined pre-incubation, post-incubation, and after the cell suspensions were prepared from Groups 1 to 7.

8. **Dicamba concentration analysis:** The supernatant of the intracellular pH aliquots from the duodenum preparations were stored at −30°C to −10°C. In addition to this, the mincing buffer remaining after the 30 minutes incubations also was stored at −30°C to −10°C. It was stated that the concentration analyses would be performed under a separate study.

9. **Comet test:** Single cell suspensions were prepared as described above. Comet slides were prepared by first dipping glass slides into 1% normal melting point agarose and allowing them to air dry. The single cell suspensions were diluted (diluent not reported) and mixed with an appropriate volume of 0.5% low melting point agarose. The diluted suspension in agarose was applied (75 µL) to the pre-dipped slide and a cover slip was applied. After the agarose solidified, the cover slips were removed. For cell suspensions prepared by Method 2, the slides were placed in lysis buffer (not defined) protected from light at 2-8°C overnight. For cell suspensions prepared by Method 1, the slides were placed in lysis buffer one hour after preparation of the first slide and refrigerated overnight. The following day, the slides were rinsed with electrophoresis buffer (not defined) and placed on a horizontal electrophoresis unit containing chilled electrophoresis buffer. Additional buffer was added to cover the surfaces of the slides and the slides were allowed to incubate for 20 minutes to allow the cellular DNA to unwind. The slides were electrophoresed at 18 V with a starting current of approximately 300 mA (0.7-1.0 V/cm) for 30 minutes. It was stated that the buffer temperature was maintained at 4-9°C during unwinding and electrophoresis. When electrophoresis was complete, the slides were removed, rinsed three times for five minutes each with neutralization buffer, and stored refrigerated and protected from light in humidified boxes. The slides were stained with a nucleic acid-specific dye (SYBR GOLD®) and examined with a fluorescence microscope linked to a CCD camera and image analysis system (Perceptive Instruments COMET IV™).

   The slides were first examined for signs of toxicity including background cellular debris and/or increased incidence of excessively damaged cells (hedgehog cells); these cells were excluded from analysis along with cells that had unusual staining artefacts. Fifty cells/slide were scored to yield 150 cells/tissue/rat (Reviewers assume three slides/tissue/rat). The extent of DNA migration (*i.e.*, damage) was measured by determining the % tail intensity defined as the fluorescence detected in the tail that is proportional to the amount of DNA that moved from the head region to the (comet) tail.

10. **Microscopic pathology:** Duodenum sections were incubated in ice-cold mincing solution for 30 minutes (as in Method 1), then fixed in 10% buffered formalin, routinely processed, and stained with either hematoxylin and eosin or active caspase 3. Slides from Groups 1-7 were examined microscopically for cytotoxicity, necrosis, or apoptosis.

II. RESULTS

A. CLINICAL SIGNS OF TOXICITY: There were no clinical signs of toxicity observed in any group. All rats survived to scheduled euthanasia.

B. BODY WEIGHTS: Body weight data are presented in Table 2. All rats in all groups lost minor amounts of weight (−4 g to −11 g) between administration of the second dose and euthanasia. These losses did not affect the results of the study.

TABLE 2. Mean (± SD) body weights (g) in rats administered two 75 mg/kg/day doses of dicamba via oral gavage 24 hours apart. [a]

Dose (mg/kg/day)	Time of euthanasia (hours post-dose) [b]	Day 1	Day 2	Day 2 euthanasia
0	2	232 ± 6.2	239 ± 5.1	235 ± 5.5
75	0.5	233 ± 9.3	238 ± 12.5	233 ± 12.4
75	1	236 ± 6.7	242 ± 6.7	235 ± 6.4
75	2	231 ± 15.5	235 ± 14.6	231 ± 14.4
75	4	234 ± 13.0	237 ± 11.6	233 ± 11.5
75	6	243 ± 11.3	250 ± 12.3	241 ± 10.6
75 [c]	2	226 ± 4.0	228 ± 4.2	223 ± 5.1
EMS 200 [d]	3	---	251 ± 3.5	240 ± 3.2

a Data were obtained from Appendix 1 on page 48 of MRID 51129103. N = 3.
b Time after the second or final dose on Day 2.
c Rats were administered three doses of 25 mg/kg at 30-minute intervals on two consecutive days.
d EMS = ethyl methanesulfonate, single 200 mg/kg dose.
--- No data.

C. COMET TEST: Percentage tail intensity and hedgehog cell data are presented in Tables 3 and 4.

1. % Tail intensity

a. Method 1: Tissue samples obtained at the 0.5-, 4-, and 6-hour time points from the rats administered two 75 mg/kg/day doses, and tissue samples obtained at 2 hours from the rats administered three 25 mg/kg doses all displayed increased mean and mean median % tail intensity that was greater than the 95% confidence limits of the historical control data. Tissue samples obtained at the 1- and 2-hour time points were similar to control. Positive control samples displayed marked increases in mean and mean median % tail intensity.

b. Method 2: Tissue samples from all time points from all treated rats displayed increased mean and mean median % tail intensity that was greater than the 95% confidence limits of the historical control data. Positive control samples were not collected by using method 2.

TABLE 3. Mean (± SD) % tail intensity and median % tail intensity in rats administered two 75 mg/kg/day doses of dicamba via oral gavage 24 hours apart. [a]					
Dose (mg/kg/day)	**Time of euthanasia (hours post-dose)**	**% intensity**		**Median % intensity**	
		Method 1	**Method 2**	**Method 1**	**Method 2**
0	2	2.23 ± 0.5	2.57 ± 0.2	0.35 ± 0.1	0.9 ± 0.1
75	.5	**15.19 ± 0.3**	**6.51 ± 0.9**	**11.29 ± 0.9**	**3.08 ± 1.1**
75	1	3.69 ± 0.4	**7.72 ± 1.7**	0.98 ± 0.6	**4.10 ± 1.3**
75	2	3.03 ± 0.6	**8.36 ± 0.8**	0.77 ± 0.3	**3.59 ± 1.4**
75	4	**11.58 ± 1.8**	**8.79 ± 4.2**	**7.08 ± 1.5**	**5.01 ± 2.5**
75	6	**15.68 ± 4.6**	**7.54 ± 4.2**	**11.68 ± 5.0**	**3.83 ± 2.8**
75 [b]	2	**10.47 ± 3.7**	---	**6.08 ± 3.8**	---
EMS 200 [c]	3	**36.58 ± 8.3**	---	**33.26 ± 10.0**	---

a Data were obtained from Table 1 on page 34 of MRID 51129103. N = 3.
b Rats were administered three doses of 25 mg/kg at 30-minute intervals on two consecutive days.
c EMS = ethyl methanesulfonate, single 200 mg/kg dose.
Bold values are >95% control limit of laboratory historical control database.
--- No data.

2. Hedgehog cells

a. Method 1: Tissue samples obtained at the 0.5-, 4-, and 6-hour time points from the rats administered two 75 mg/kg/day doses, and tissue samples obtained at 2 hours from the rats administered three 25 mg/kg/day doses all displayed markedly increased numbers of hedgehog cells. Tissue samples obtained at the 1-hour time point displayed a slight increase in hedgehog cells. Tissue samples from the 2-hour time point, control, and positive control groups had no hedgehog cells.

b. Method 2: Tissue samples from all time points from all treated rats displayed increased numbers of hedgehog cells; however, hedgehog cells also were observed in the vehicle control group. Positive control samples were not collected by using method 2.

TABLE 4. Mean (± SD) hedgehog cell data in rats administered two 75 mg/kg/day doses of dicamba via oral gavage 24 hours apart. [a]			
Dose (mg/kg/day)	**Time of euthanasia (hours post-dose)**	**# of Hedgehog cells**	
		Method 1	**Method 2**
0	2	0.0 ± 0.0	8.3 ± 1.2
75	.5	35.3 ± 8.5	10.3 ± 4.5
75	1	1.3 ± 1.5	14.0 ± 2.6
75	2	0.0 ± 0.0	11.0 ± 2.0
75	4	39.3 ± 6.7	15.7 ± 11.6
75	6	50.7 ± 9.7	16.0 ± 10.1
75 [b]	2	20.7 ± 14.3	---
EMS 200 [c]	3	0.0 ± 0.0	---

a Data were obtained from Table 3 on page 39 of MRID 51129103. N = 3.
b Rats were administered 3 doses of 25 mg/kg at 30-minute intervals on two consecutive days.
c EMS = ethyl methanesulfonate, single 200 mg/kg dose.
--- No data.

3. Intracellular pH: It was stated that the differences in intracellular pH detected by using kit 1 were not substantial but that an impact on intracellular pH levels could be detected. The Reviewers do not consider this data useful to make any conclusions regarding intracellular pH. Responses were described as no fluorescence, poor quality, low intensity, or slight intensity; quantification of these descriptions was not possible.

4. **Mincing solution pH and osmolality measurements:** Measurements of pH and osmolality of the mincing solution post-incubation or following preparation of the cell suspensions were generally similar to the values obtained pre-incubation.

5. **Microscopic pathology:** There were no effects of treatment noted during the microscopic examinations.

III. DISCUSSION and CONCLUSIONS

A. **INVESTIGATORS' CONCLUSIONS:** This study confirmed previous data, that Dicamba techn. (BAS 183 H; SAN837 techn.) induces DNA damage under the described circumstances. However, it remains unclear whether this damage is caused by a direct interaction with the host DNA or is a product of indirect effects such as exposure to low intracellular pH. Several parameters such as the intracellular pH determination, the length of the exposure periods and the split dosing suggest the latter mechanism. However, the data are too inconclusive for a definitive outcome.

B. **REVIEWER COMMENTS:** The Reviewers agree with the Investigators' Conclusions that the data generated in the present study are not conclusive.

Comet test data showed increased % tail intensity accompanied by increased hedgehog cells across some, but not all, time points. The positive control generated data consistent with expected findings. Intracellular pH data was subjective, not quantifiable, and inconclusive. Mincing solution pH and osmolality were not affected by incubation times or cell suspension preparation. There were no microscopic findings of cytotoxicity, necrosis, or apoptosis.

This study is classified as **acceptable / non-guideline**.

C. **STUDY DEFICIENCIES:** The following deficiencies were noted:

- Concentration analysis of the formulations were not reported.
- Methods for caspase 3 staining procedures were not reported.